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Article

Serotypes and *Stx2* subtyping of Shiga toxin producing *Escherichia coli* isolates from cattle carcasses and feces

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Abstract:

Shiga toxin *E. coli* (STEC) is an important pathogen responsible for foodborne illness, this have been related with epidemic outbreaks in the past, mainly because of consumption of bovine meat. The objective of this study was identify the serotypes and Stx2 subtypes and associate them with their possible epidemiology. There were analyzed a total of 65 isolates from the collection of the Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia of the Universidad Autónoma del Estado de México, from carcasses and feces of bovines at three different Municipal slaughterhouses. The identification of *Stx2* gene by PCR at final point, sequencing and analyzed with the help of BLAST software. There were found O157:H7, O70:H16, O91:H10, O112ac:H2, O128ac:H26 serotypes, which have been reported to be present at infectious outbreaks previously by foodborne worldwide; 63.07% (41/65) of the *Escherichia coli* strains got amplified for Stx2 and after BLAS analysis it was confirmed its presence and a hypothetic protein. The presence of this serotypes in combination with different subtype's, Stx2a, Stx2c, Stx2d, in carcasses and feces of bovine in must be considered as a potential risk for diseases an important problem of the public health.

Key words: Cattle carcasses, *Escherichia coli*, Serotyping, stx2.

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Introduction

Some pathogenic serotypes of *Escherichia coli* (*E. coli*) are a cause of food borne diseases, and are mainly associated with the consumption of beef, unpasteurized milk, apple juice, yogurt, cheese, fresh water and raw salads[1,2]. For *E. coli* to cause a disease in humans a variety of conditions are required; however, there are some pathogenic strains that are considered as primary agents since they have acquired virulence factors via plasmids, transposons or bacteriophages or transmitted by mobile genetic elements (MGEs)[2]. It is important to consider that cattle are the main reservoir and the products and by-products obtained from them are considered as a source of infection of *E. coli* producing Shiga toxin (STEC)[1-4]. Currently in the municipal slaughterhouses there are health risks, the slaughterhouse sampled have a medium health risk and high sanitary risk, according to the

Guide for carrying out the sanitary diagnosis and detection of operational needs of municipal slaughterhouses of COFEPRIS (Federal Commission for the Protection against Sanitary Risks) since it mentions that approximately 18 % of the annual slaughter of cattle is carried out in establishments with high or very high health risk; this is the concern since annually, in establishments considered as having a high or very high health risk, 112,000 t of beef and if the annual per capita consumption of beef is taken into account, it would be expected that approximately a total of 7'103,300 people will consume beef produced in establishments of high or very high risk. All this probably due to poor sanitary conditions such as the lack of inadequate toilet facilities and equipment, poor sanitary habits of the workers and deficiency in the cleaning of utensils and work clothing.

An estimated of 265,000 STEC cases occur each year in the United States[5]. The main serotype involved in clinical conditions in humans is O157:H7[6], which causes about 36 % of these infections and 64 % is caused by non-O157. Public health expert's opinions are based on estimates and not real data because not all STEC infections are diagnosed and reported, as there are not approved laboratories for the isolation of non-O157[7] or the laboratories vary widely in their stool culture protocols and in their abilities to detect this organism. The Shiga toxin family includes several toxins related to Shiga toxin from *Shigella dysenteriae* that share a similar structure and biological activity, Shiga toxin nomenclature is a system based on phylogenetic sequence based relatedness of the proteins, the Stx nomenclature is designated Stx1a, Stx1c, Stx1d, Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g[8]. Stx2a can cause a greater harm than stx1; stx2a was associated with serotype O104:H4 in a foodborne outbreak in Germany and other countries that affected more than 4,000 people in 2011, 23 % of this case evolved to develop hemolytic uremic syndrome (HUS)[9]. Other serotypes with this variant are O157:H7[10] and O26:H11[11], it suggests that stx2a is directly associated with HUS[9-11]. Stx2b is often present in sheep, goats and deer, meanwhile Stx2c toxin to healthy or sick pigs[6], Stx2d toxin was identified in serotype O26 from a patient with bloody diarrhea and HUS in Germany, also it was found in cheese and cattle[12], meat and pork byproducts and wild pigs[6]. The stx2f variant has been reported in pigeons[13], but so far, reports of disease in humans are rare[8]. The objective of this study was identify the serotypes and Stx2 subtypes and associate them with their possible epidemiology, there are studies where there is a correlation between the serotype and subtype of Stx2 with the type of virulence besides the risk of associating with infections of importance.

Material and methods

Isolated background

The slaughterhouses where the carcasses and feces were sampled have a medium health risk and high sanitary risk, according to the Guide for carrying out the sanitary diagnosis and detection of operational needs of municipal slaughterhouses of COFEPRIS, during the summer sampling was carried out on slaughterhouses, where pigs, sheep and mainly cattle are slaughtered, the animals that arrive are from livestock production of municipalities adjacent to the slaughterhouses for this studies the number of animals slaughtered weekly in the 3 (A, B and C) was taken, with a sacrifice of 120, 80 and 375 respectively, 575 slaughtered bovines were accounted per week, and the sample size was obtained, considering a 2.7 % prevalence and a 95 % confidence level, by means of the finite population sample size formula; hence, the following sample size was obtained: slaughterhouse A, 8; slaughterhouse B, 5; and slaughterhouse C, 25. Three repetitions were performed in each slaughterhouse taking paired samples (114 carcasses and 114 feces). The carcass sample was taken after its washing and before refrigeration, the fecal matter samples were taken after animal evisceration.

Strains in study

There were analyzed a total of 65 isolates of *Escherichia coli*. from the collection of the Centro de Investigacion y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma del Estado de México. They were obtained from carcasses and bovine's feces from three municipal slaughterhouses (A, B and C) in the center of Mexico.

Serotyping

It was performed according to the procedure described by Orskov and Orskov[14]. It was used specific serum anti-H and anti-O (SERUNAM, Mexico) for a total of 185 somatic antigens and 56 flagellar.

DNA extraction

It was made from bacterial frozen cells at -70 °C; 20 µl of bacterial culture were first taken and suspended in 200 µl of sterile distilled water, and then incubated at 100 °C for 10 min to be used directly in PCR reactions[15].

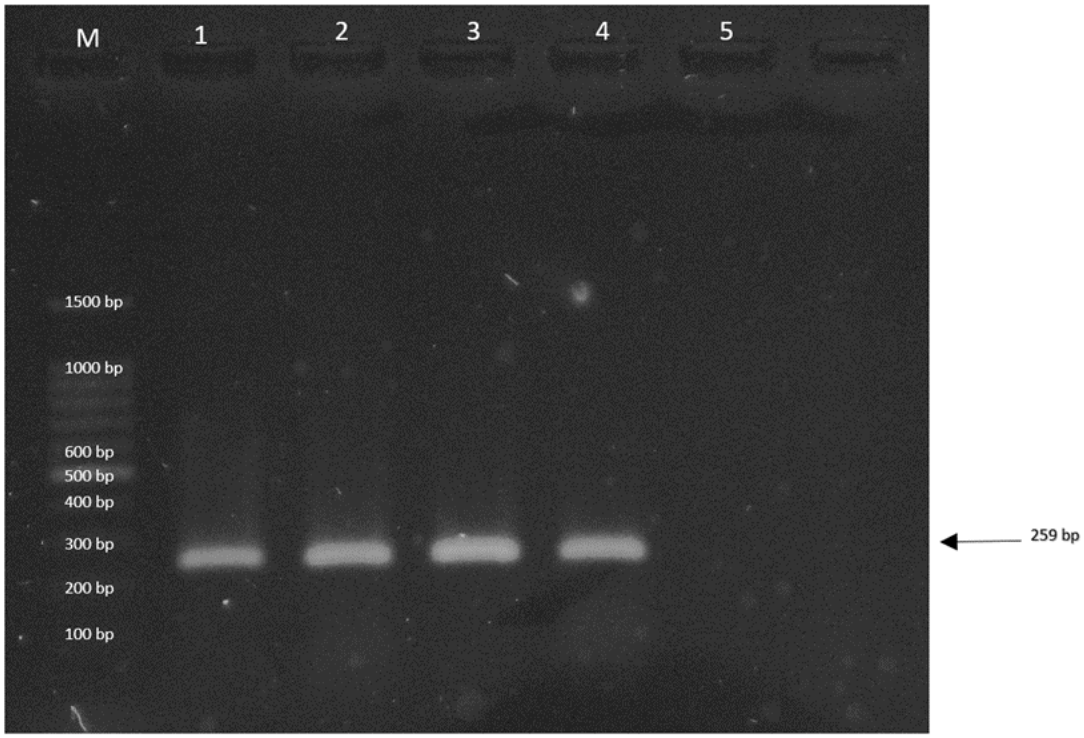
Identification of Shiga toxin 2

The following primers were employed 5´CTT CGG TAT CCT ATT CCC GG3´, 5´CTG CTG TGA CAG TGA CAA AAC GC3´[16] y 5´GGC ACT GTC TGA AAC TGC TCC 3´, 5´TCG CCA GTT ATC TGA CAT TCT G3´[17]. EDL933 strain of *E. coli* was used as positive control, the PCR conditions were described according previously mentioned authors. The products from amplification were separated by electrophoresis in agarose gels at 2%, they were visualized and captured with an image photodocumenter (UV Transilluminator UVP Model M-20E and Kodak Digital Science electrophoresis documentation and analysis system 120). Strains that tested positive for stx2 and the PCR reaction were purified using the Wizard® SV Gel kit and PCR Clean -Up System (Promega) and sequenced on an analyzer ABI PRISM 3730XL (Macrogen, Inc., Korea). The nucleotide sequence of the Stx2 toxins tested were analyzed in the Standard Nucleotide BLAST (Basic Local Alignment Search Tool).

Results

In this study 36 different serotypes were found; 12.31 % (7/41) O157:H7, 9.23 % (6/65) O22:H8, 6.15 % (4/65) O?:H7, 4.62 % (3/65) O112ac:H2, O128ac:H26, O185:H2 and OR:H7, 3.07 % (2/65) O18ac:H21, O37:H10, O103:H16, O117:H4, O147:H28, O154:H53 and O?:H51, 1.54 % (1/65) O7:H30, O8:H25, O18ac:NM, O18ac:H7, O37:H?, O40:NM, O53:H2, O65:H16, O70:H16, O91:H10, O118ac:H21, O120:H10, O136:H16, O149:H2, O172:H45, O184:H12, O185:H7, OR:H2, OR:H?, O?:H2 y O?:H11 (Figure 1, Table 1).

Figure 1: DNA amplification by PCR



Lane M Molecular weight marker: Lane 1 sample positive stx2 (O157:H7); lane 2-3 sample positive hypothetical protein (O18ac:NM and O102:H40); lane 4 positive control; lane 5 negative control.

From slaughterhouse A were identified 9 serotypes (O37:H10, O40:NM, O65:H16, O70:H16, O112:H2, O157:H7, O172:H45, 0184:H12 and O?:H2) from 10 isolates from which in the 50 % (5/10) the stx2 toxin was identified. In slaughterhouse B from the 10 isolates, were observed 9 serotypes (O18:H7, O112:H2, O120:H10, O128:H26, O185:H7, OR:H2, ORH?, O?:H7 and O?H11) 20 % (2/10) of them tested positive for stx2. At slaughterhouse C, from the 45 isolates were found 23 different serotypes; 64.4 % tested positive for stx2. In this place was found that serotypes got amplified with primers but, after sequencing, only 17.8 % (11/45) was identified to be a hypothetica l protein (Figure 1, Table 1).

Table 1: Identification of *Stx2* gene in shiga toxin producing *Escherichia coli* and Serotypes isolated from cattle carcasses

Serotype	Isolate	Sample type*	Stx2	Subtypes	Accession number
O8:H25	43	Carcass (C)	+	Stx2a	KT356574
O18ac:NM	49	Carcass (C)	+	HP	KT356580
O18ac:H7	11	Carcass (B)		Negative	
O18ac:H21	39	Carcass (C)	+	Stx2a	KT356571
O22:H8 (3)	45	Carcass (C)	+	Stx2a	KT356576
	47	Carcass (C)	+	Stx2a	KT356578
	50	Carcass (C)	+	Stx2d	KT356581
O37:H10	6	Carcass (A)		Negative	
O40:NM	7	Carcass (A)	+	Stx2a	KT356555
O65:H16	1	Carcass (A)	+	Stx2c	KT356546
O91:H10	27	Carcass (C)	+	Stx2c	KT356564
O102:H40	22	Carcass (C)	+	HP	KT356560
O112ac:H2 (2)	2	Carcass (A)	+	Stx2a	KT356548
	19	Carcass (B)		Negative	
O118ac:H21	21	Carcass (C)	+	Stx2c	KT356559
O120:H10	20	Carcass (B)		Negative	
O128ac:H26 (2)	16	Carcass (B)		Negative	
	41	Carcass (C)	+	Stx2c	KT356573

O136:H16	52	Carcass (C)	+	Stx2c	KT356582
O149:H2	35	Carcass (C)	+	HP	KT356567
O154:H53 (2)	32	Carcass (C)		Negative	
	33	Carcass (C)			
O157:H7 (5)	23	Carcass (C)	+	Stx2c	KT356561
	3A	Carcass (C)	+	Stx2c	KT356551
	4A	Carcass (C)	+	Stx2c	KT356552
	5A	Carcass (C)	+	Stx2c	KT356553
	6A	Carcass (C)	+	Stx2c	KT356554
O172:H45	9	Carcass (A)		Negative	
O185:H2	29	Carcass (C)		Negative	
OR:H7 (3)	1A	Carcass (C)	+	Stx2a	KT356547
	2A	Carcass (C)	+	Stx2a	KT356549
	C	Carcass (C)	+	Stx2a	KT356585
ONT:H2	4	Carcass (A)		Negative	
ONT:H7 (2)	14	Carcass (B)		Negative	
	G	Carcass (C)	+	HP	KT356586
ONT:H11	12	Carcass (B)		Negative	
ONT:H51	25	Carcass (C)	+	Stx2a	KT356563

( ) Slaughter plant A, B, or C.
HP= hyphotetic protein; NM= no movile; NT= nontypeable; R= rough.

In this study, 63.07 % (41/65) of the strains of *Escherichia coli* amplified to stx2, after analysis of its sequence in BLAST it was confirmed that 71.5 % (33/41) were Stx2; also strains amplified with primers were found, but after the analysis of the sequence performed in BLAST were identified that 19.5 % (8/41) was a hypothetical protein and were submitted to GenBank database whose access numbers are KT356546, KT356547, KT356548, KT356549, KT356550, KT356551, KT356552, KT356553, KT356554, KT356555, KT356556, KT356557, KT356558, KT356559, KT356560, KT356561, KT356562, KT356563, KT356564, KT356565, KT356566, KT356567, KT356568, KT356569, KT356570, KT356571, KT356572, KT356573, KT356574, KT356575, KT356576, KT356577, KT356578, KT356579, KT356580, KT356581, KT356582, KT356583, KT356584, KT356585, KT356586 (Table 1).

Discussion

Escherichia coli has been associated with various pathological conditions[4]; from 1980 to the present have been recognized over 6,600 entries representing 1,152 STEC serotypes[18] some of these are implicated in outbreaks of sporadic diseases in humans and cattle, and their products act as a primary source of pollution[1,3]. In Mexico there are few reports of *E. coli* serotypes, in this study 36 different serotypes were found. Amézquita-López *et al*[19] identified 19 serotypes from feces of domestic animals in rural farms in Culiacan Sinaloa, Mexico; the main serotype found was O157:H7 with 43.1 % (28/65) from which 57 % (16/28) were from bovines. Other serotypes found were O8:H19, O15:NT, O73:NT, O75:H8, O168:NT, in this study the serotype O157:H7 was also the most frequent (12.31 %) but none of the serotypes reported by Amézquita-López *et al*[19] were identified. In Argentina and Germany the most important serotype in cattle and its byproducts is O178:H19[4] and represents an emerging serotype frequently isolated in outbreaks of hemorrhagic colitis and hemolytic uremic syndrome in humans[20]. Other studies found serotypes O70:H16, O91:H10, O112ac:H2 and O128ac:H26 as a cause of HUS in Germany[6], in this study these same serotypes were found so they are an important finding for public health in Mexico. Fernández *et al*[21] identified more frequently O113:H21, O130:H11 and O178:H19 serotypes in bovine's feces in Argentina. In study in Germany identified O113:H21, O22:H8, O174:H21, O178:H19, O113:H4 and O91:H14 serotypes and they mentioned that these are the most common serotypes in cattle in the world[6], in France reported the serotypes O157:H7, O26:H11, O103:H2, O111:H8 and O145:H28 obtained from bovine feces in slaughterhouses[22]. The most frequent was O157:H7 with a percentage up to 1.2 %, which is lowest than the percentage obtained in this study (9.23 %), this difference may arise by the particular conditions of the slaughterhouse or the period in which the study was done. In the serogroup O128, although it is most common isolated from sheep, it has been found in human

patients, healthy cattle and beef; in this study it was found in carcasses and bovine's feces with the presence of Stx2c[18]. Today this serogroup is no longer in a specific species. The distribution and frequency of the serotypes and pathotypes may vary considerably from region to region, in that sense serotypes may vary in their pathogenicity or may become emerging serotypes[23].

Shiga toxins are considered the major virulence factors in the development of HUS[2]. Friesema *et al*[13] mentioned that the presence of subtypes stx2 showed a difference at the point of clinical manifestations in humans, in this study, 63.07 % (41/65) of the strains of *Escherichia coli* amplified to stx2, after analysis of its sequence in BLAST it was confirmed that 71.5 % (33/41) were Stx2; also strains amplified with primers were found, but after the analysis of the sequence performed in BLAST were identified that 19.5 % (8/41) was a hypothetical protein. In a study from China found Stx2b, Stx2c and Stx2e in beef from supermarkets[3]. In the O22 serogroup isolated from beef was found stx2b toxin, these results are significant because the same serotype found could have this same variety; however other study mentioned that this variety it is capable of potential cause of HUS in serogroup O26[11]. In report from retail raw meats in China identified the toxin Stx2a in O40 serogroup, also they reported serogroup O91 from pork and lamb with presence of Stx2e and Stx2b respectively[3], and in this study the same serogroup may also have those varieties although this isolate was obtained from bovine's carcasses. In a study from 210 patients with HUS of different regions of Germany reported the serotype O91:H2[24] and compare its virulence with O91:H10 as the main cause of HUS. Bai *et al*[3] believe that having a serotype with Stx2 toxin present in carcasses from cattle for human consumption could be a major risk also identified serogroup O103 without the presence of Stx2 toxin, in this study two isolates of the same serogroup were obtained but only one had the presence of Stx2, this serogroup is of importance since it has been reported to cause HUS[20]. Other study found the serogroup O128 with Stx2b[3], in this study in the same serogroup was found the Stx2c. in Germany investigated for the presence of STEC during routine diagnostic work in the Institute for Hygiene and Microbiology and identified non-O157 Stx2c strains and mentioned that is more likely to find HUS in this variant, while the presence of Stx2d can manifest itself in a milder form[25]. In Turkey have associated an increased cytotoxicity in developing HUS from O157:H7 strain with the presence of Stx2c[26]; this agrees with another study in Switzerland where they associate the presence of stx2a/stx2d/stx2c in patients with diarrhea, and HUS[27], in this study, in the same serotype was identified the toxin stx2c and it could be a risk factor to cause disease.

From slaughterhouse A were identified 9 serotypes (O37:H10, O40:NM, O65:H16, O70:H16, O112:H2, O157:H7, O172:H45, 0184:H12 and O?:H2) from 10 isolates from which in the 50 % (5/10) the stx2 toxin was identified. In slaughterhouse B from the 10 isolates, were observed 9 serotypes (O18:H7, O112:H2, O120:H10, O128:H26, O185:H7, OR:H2, ORH?, O?:H7 and O?H11) 20 % (2/10) of them tested positive for Stx2. At Slaughterhouse C, from the 45 isolates were found 23 different serotypes; 64.4 % tested positive for Stx2. In this place was found that serotypes got amplified with primers but, after sequencing, only 17.8 % (11/45) was identified to be a hypothetical protein (Table 1).

Foodborne diseases (FD) are produced by the ingestion of food or water, contaminated with biological agents, which affects the health of the consumer, which can be caused by inadequate handling of food at any stage of its production chain. In Mexico gastrointestinal diseases occupy the second place according to the Ministry of Health with 6'106,572 cases accumulated in 2017, considering that of these 5'606,759 have not been associated with an etiological agent, in the Central Mexican High Plateau 94,081 cases have been reported in this area, although in Mexico epidemiological studies do not reveal in all cases the foods associated with the FD, nor the microorganisms involved, much less if the cases of HUS are related to *Escherichia coli*, only the General Directorate of Epidemiology of the Ministry of Health records a significant number of cases each year, with rates that reached between 4,859 cases per 100 thousand inhabitants. Another estimate indicates that, if there are about 5 million annual cases of diarrheal diseases in Mexico, and a conservative adjustment indicates that only 50 % are caused directly by food, with an underreporting of 1 per 100 episodes, the actual number of cases It would be around 250 million events per year, equivalent to 2.5 episodes per person per year. What leads to a significant economic impact, causing a decrease in the productivity of people due to absenteeism or poor performance at work, economic losses to the country due to an increase in the demand for medicines, medical and hospital services, a negative impact on the tourism and in the development of national and international trade. So a study of the Pan American Institute for Food Protection and Zoonoses (INPPAZ), estimated the economic losses by FD in Mexico, in 1.1 trillion dollars only by reduction of productivity.

This work demonstrates the importance of bovine carriers of *E. coli* with serotypes of importance in public health, that when it is inadequate management in slaughterhouses can be contaminated the carcasses so there must be the need to improve the process to obtain information on meat, therefore a strategy to reduce the risk of infections by serotypes of this language in humans would be to reduce the prevalence in livestock, as well as improve management in slaughterhouses.

Conclusions and implications

It was showed the presence of a variety of STEC serotypes in bovine's carcasses and feces from municipal slaughterhouses in Mexico. It was also shown the presence of important serotypes for human and animal health that have present the toxin stx2 which may have the potential to cause HUS, so it is considered as an important risk factor that can trigger human diseases. A slaughterhouse deals with the transformation of one or several kinds of beef cattle for human consumption through a series of basic and determinant stages in the quality of the meat and there is a responsible authority that has the legal power to establish and make comply with the requirements on meat hygiene. There are programs on meat hygiene and its main objective is the protection of public health, in addition to the fact that they base their decisions on the scientific evaluation of the possible risks to human health, where they consider all the food dangers identified in research, monitoring and other relevant activities, so that food hygiene is defined as all the conditions and measures necessary to ensure the safety and suitability of food in all steps of the food production chain. The safety of a production is not guaranteed by the bacteriological examination of the finished product, but through a strict compliance of each one of the stages of the process of obtaining the meat.

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